Form PTO-1390 (REV 10-94) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	
641050.90021	
U.S. APPLICATION NO. (If known, see 37 C.F.R. 1.5)	
09/530233	

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/CA98/01016	29 October 1998	29 October 1997

TITLE OF INVENTION: DNA ENCODING A HUMAN PROTON-GATED ION CHANNEL AND USES THEREOF

APPLICANT(S) FOR DO/EO/US: SEGUELA, Philippe; BABINSKI, Kazimierz

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [X] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US)
6. [] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been transmitted by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.
8. [ ] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [X] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 in included.
13. [X] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [X] Other items or information: Verified Statements (Declaration) Claiming Small Entity Status - Small Business Concern of Applicants Seguela and Babinksi; Postcard Receipt

EXPRESS MAIL NO. EL549847444US

QB

(January 1997)

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
09/530233	PCT/CA98/01016	641050.90021

17. [X] The following fees are submitted:

	CALCULATIONS (PTO USE ONLY)	
BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)): Search Report has been prepared by the EPO or JPO $840.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) $670.00		
No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2)) $690.00		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO ..... $ 970.00		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4) $96.00		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 840.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$	

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	18 -20 =		X $18.00	$	
Independent claims	1 -3 =		X $78.00	$	
MULTIPLE DEPENDENT CLAIM(S) (if applicable)	6		X $260.00	$	

TOTAL OF ABOVE CALCULATIONS =	$ 840.00	
Reduction by 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).	$ 420.00	
SUBTOTAL =	$ 420.00	
Processing fee of **$130.00** for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.429(f)). +	$	
TOTAL NATIONAL FEE =	$	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +	$ 40.00	
TOTAL FEES ENCLOSED =	$ 460.00	
	Amount to be: refunded	$
	charged	$

a. [ ] A check in the amount of $_____ to cover the above fees is enclosed.

b. [X] Please charge my Deposit Account No. 17-0055 in the amount of $ 460.00 to cover the above fees.
A duplicate copy of this sheet is enclosed.

c. [X] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 17-0055. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

SIGNATURE Jean C. Baker

NAME Jean C. Baker

REGISTRATION NUMBER Reg. No. 35,433

QB

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of:	**SEGUELA et al.**	Docket No.:	641050.90021
Serial No.:	**Unassigned**	Filed:	**Concurrently herewith**
Int'l appln No.:	**PCT/CA98/01016**	Int'l filing date:	**29 October 1998**
Title:	**DNA ENCODING A HUMAN PROTON-GATED ION CHANNEL AND USES THEREOF**		

****

PRELIMINARY AMENDMENT

Box PCT
Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

In connection with the above-identified application filed herewith, please enter the following preliminary amendment:

IN THE CLAIMS:

Please cancel claims 1-15 without prejudice.

Please add the following new claims:

–16. A proton-gated cation channel having the amino acid sequence of SEQ ID NO:1 or a variant of that sequence having at least 85% identity therewith.

17. A channel according to Claim 16 which forms a homopolymeric channel.

18. A channel according to Claim 16 in combination with another cation channel sub-unit which together form a heteropolymeric channel.

19. A channel according to Claim 18 wherein the other cation channel belongs to the degenerin/ENaC channel superfamily.

20. A channel according to Claim 18 wherein the other cation channel is a P2X ATP-gated channel.

21. A channel according to Claim 20 wherein the other cation channel sub-unit of P2X2 ATP-gated channel sub-unit.

22. A nucleic acid encoding a proton-gated cation channel as defined in Claim 16.

23. A nucleic acid encoding a proton-gated cation channel having the amino acid sequence of SEQ ID NO:1 or a variant of that sequence having at least 85% identity therewith and another cation channel sub-unit as defined in Claim 21.

24. A recombinant vector comprising a nucleic acid as defined in Claim 22.

25. A recombinant vector comprising a nucleic acid as defined in Claim 23.

26. A recombinant host cell comprising the nucleic acid of Claim 22.

27. A recombinant host cell comprising the nucleic acid of Claim 23.

28. A method of producing a proton-gated cation channel, which comprises the steps of:

incubating the host cell of Claim 26 under conditions which allow the DNA sequence to be expressed, and isolating the protein from the host.

29. A method of producing a proton-gated cation channel, incubating the host cell of Claim 27 under conditions which allow the DNA sequence to be expressed, and isolating the protein from the host.

30. A method for screening compounds useful as proton-gated cation channel ligands, which comprises the steps of:

contacting a cell bearing at its surface a channel as defined in Claim 16 with said compounds; and

selecting the compounds that bind to said channel as ligands.

31. A method of screening compounds useful as proton-gated cation channel ligands, which comprises the steps of:

contacting a cell bearing at its surface a channel as defined in Claim 21 with said compounds; and

selecting the compounds that bind to said channel as ligands.

32. A composition or kit for screening compounds useful as proton-gated cation channel ligands, which comprises a cell population expressing a channel as defined in Claim 16 at its surface, and auxiliary components assisting the binding and the detection of the binding of said ligands to said channel.

33. A composition or kit for screening compounds useful as proton-gated cation channel ligands, which comprises a cell population expressing a channel as defined in Claim 21 at its surface, and auxiliary components assisting the binding and the detection of the binding of said ligands to said channel.--

Remarks

The above amendments are being made to eliminate multiple dependencies in the claims and incorporate new claims 16-33 into the application.

No fee is believed necessary to enter this amendment. However if a fee is necessary, please charge Deposit Account 17-0055.

Applicant respectfully requests that the preliminary amendment described herein be entered into the record prior to examination and consideration of the above-identified application.

QUARLES & BRADY LLP

BY: Jean C. Baker

Jean C. Baker
Registration No. 35,433

Date: April 26, 2000

QUARLES & BRADY
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Milwaukee WI 53202-4497
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(414) 277-5709

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) AND 1.27 (c)) - SMALL BUSINESS CONCERN	Docket No.

Serial No.	Filing Date	Patent No.	Issue Date

Applicant/
Patentee: **Philippe SÉGUÉLA and Kazimierz BABINSKI**

Invention: **DNA ENCODING A HUMAN PROTON-GATED ION CHANNEL AND USES THEREOF**

I hereby declare that I am:

- ☐ the owner of the small business concern identified below:
- ☒ an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN: **Antalium**

ADDRESS OF CONCERN: **1550, rue Metcalfe, Bureau 502, Montréal (Québec) CANADA H3A 1X6**

I hereby declare that the above-identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under Section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the above identified invention described in:

- ☒ the specification filed herewith with title as listed above.
- ☐ the application identified above.
- ☐ the patent identified above.

If the rights held by the above-identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed on the next page and no rights to the invention are held by any person, other than the inventor, who could not qualify as an independent inventor under 37 CFR 1.9(c) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

Each person, concern or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:

☐ no such person, concern or organization exists.
☒ each such person, concern or organization is listed below.

FULL NAME **McGill University**
ADDRESS **3550 University Street, Montréal, Québec, CANADA H3A 2A7**

☐ Individual ☐ Small Business Concern ☒ Nonprofit Organization

FULL NAME
ADDRESS

☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

FULL NAME
ADDRESS

☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

FULL NAME
ADDRESS

☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING: **Kazimierz BABINSKI**
TITLE OF PERSON SIGNING OTHER THAN OWNER: **President and CEO**
ADDRESS OF PERSON SIGNING: **1550, rue Metcalfe, Bureau 502, Montréal (Québec) CANADA H3A 1X6**

SIGNATURE: Kazimierz Babinski DATE: April 14, 2000

VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) AND 1.27 (d)) - NONPROFIT ORGANIZATION	Docket No.

Serial No.	Filing Date	Patent No.	Issue Date

Applicant/
Patentee: **Philippe SÉGUÉLA and Kazimierz BABINSKI**

Invention: **DNA ENCODING A HUMAN PROTON-GATED ION CHANNEL AND USES THEREOF**

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

NAME OF ORGANIZATION: **McGill University**
ADDRESS OF ORGANIZATION: **3550 University Street**
Montréal, Québec
CANADA
H3A 2A7

TYPE OF NONPROFIT ORGANIZATION:

- ☒ University or other Institute of Higher Education
- ☐ Tax Exempt under Internal Revenue Service Code (26 U.S.C. 501(a) and 501(c)(3))
- ☐ Nonprofit Scientific or Educational under Statute of State of The United States of America
  Name of State: Citation of Statute:
- ☐ Would Qualify as Tax Exempt under Internal Revenue Service Code (26 U.S.C. 501(a) and 501(c)(3)) if Located in The United States of America
- ☐ Would Qualify as Nonprofit Scientific or Educational under Statute of State of The United States of America if Located in The United States of America
  Name of State: Citation of Statute:

I hereby declare that the above-identified nonprofit organization qualifies as a nonprofit organization as defined in 37 C.F.R. 1.9(e) for purposes of paying reduced fees to the United States Patent and Trademark Office regarding the invention described in:

- ☒ the specification to be filed herewith.
- ☐ the application identified above.
- ☐ the patent identified above.

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the above-identified nonprofit organization are not exclusive, each individual, concern or organization having rights to the invention is listed on the next page and no rights to the invention are held by any person, other than the inventor, who could not qualify as an independent inventor under 37 CFR 1.9(c) or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e).

Each person, concern or organization to which I have assigned, granted, conveyed, or licensed or am under an obligation under contract or law to assign, grant, convey, or license any rights in the invention is listed below:

☐ no such person, concern or organization exists.
☒ each such person, concern or organization is listed below.

FULL NAME **Antalium**
ADDRESS **1550, rue Metcalfe, Bureau 502, Montréal (Québec) CANADA H3A 1X6**

☐ Individual ☒ Small Business Concern ☐ Nonprofit Organization

FULL NAME
ADDRESS

☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

FULL NAME
ADDRESS

☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

FULL NAME
ADDRESS

☐ Individual ☐ Small Business Concern ☐ Nonprofit Organization

Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. (37 CFR 1.27)

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. (37 CFR 1.28(b))

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING: **Dr. Alex Navarre, Director, Office of Technology Transfer**
TITLE IN ORGANIZATION: **McGill University**
ADDRESS OF PERSON SIGNING: **3550 University Street, Room 101**
Montreal, Quebec, Canada H3Z 2A7

SIGNATURE: _____ DATE: 17 - 04 - 2000

TITLE OF THE INVENTION

DNA ENCODING A HUMAN PROTON-GATED ION-CHANNEL AND USES THEREOF

FIELD OF INVENTION

In mammals, the pH of the extracellular compartment, including interstitial fluids and blood, is strictly regulated and maintained at a constant value of 7.4. Acid sensing is a specific kind of chemoreception that plays a critical role in the detection of nociceptive pH imbalances occurring, for example, in conditions of cramps, trauma, inflammation or hypoxia (Lindahl, 1974). In mammals, a population of small-diameter primary sensory neurons in the dorsal root ganglia and trigeminal ganglia express specialized pH-sensitive surface receptors activated by increase of extracellular proton concentration (Bevan and Yeats, 1991). Acid sensitivity of sensory as well as central neurons is mediated by a family of proton-gated cation channels structurally related to *C. elegans* degenerins and mammalian epithelial sodium channels. This invention relates to these Acid Sensing Ion Channels (ASIC), particularly to a non-inactivating proton-gated channel, named hASIC3, its association with other channel subunits and uses thereof.

BACKGROUND OF INVENTION

Tissue acidosis is associated with a number of painful physiological (e.g. cramps) and pathological conditions (e.g. inflammation, intermittent claudication, myocardial infarction). Experimentally, similar painful events can be reproduced by infusing low pH solutions into skin or muscle. Furthermore, the prolonged intradermal infusion of low pH solutions can mimic the characteristic hyperalgesia of chronic pain. To further characterize the effects of protons and their relation to pain, low pH solutions were applied to patch-clamped central and peripheral sensory neurons. Inward currents were induced when pH was dropped to acidic values, providing evidence for the existence of proton-activated ion channels. Several types of native currents were observed in sensory neurons from rat and human trigeminal and dorsal root ganglia:

- rapidly inactivating currents;
- non-inactivating currents; and
- biphasic currents displaying a rapidly inactivating current followed by a non-inactivating current.

Other differences regarding ion selectivities were also reported. These results suggested the existence of several proton-gated ion channels. The prolonged pain induced by tissue acidification is most likely associated with a non-inactivating proton-gated ion channel.

Cloned proton-gated ion channels

Three classes of mammalian proton-gated cation channels have recently been cloned in rat and named « ASIC » for Acid Sensing Ion Channels. Sequence analysis identifies them as members of the DEG/ENaC superfamily of ion channels. The putative membrane topology of ASIC receptors predicts two transmembrane spanning domains with both N- and C-termini in the intracellular compartment, as shown for the epithelial sodium channels (ENaC). The published ASIC receptors are described below:

1) **ASIC1A** (first reported as ASIC), displays a rapidly inactivating current with a pH_{50} of 6.2 (Waldmann R. et al., *Nature* 1997; **386**: 173-7). N.B.: pH_{50} indicates the pH at which peak inward current equals half the maximal value.

2) **ASIC1B** (initially reported as ASIC-β), is a splice variant of ASIC1A where the first 185 amino acids of ASIC1A are replaced by a distinct new sequence of 172 amino acids. ASIC1B shows similar current kinetics and pH_{50} as those observed for ASIC1A (Chen C-C et al., *Proc Natl Acad Sci* 1998; **95**: 10240-5).

3) **ASIC2A** (first reported as MDEG, then MDEG1), displays a slowly inactivating current with a pH_{50} of 4.05 (Waldmann R. et al., *J Biol Chem* 1996; **271**: 10433-6).

4) **ASIC2B** (first reported as MDEG2) is a splice variant of ASIC2A where the first 185 amino acids are replaced by a distinct new sequence of 236 amino acids (Lingueglia E. et al., *J Biol Chem* 1997; **272**: 29778-83). When expressed in heterologous expression systems, ASIC2B does not appear to be activated by protons.

5) **DRASIC**, which displays a biphasic current where the rapidly inactivating component has a pH_{50} of 6.5 and the non-inactivating component a pH_{50} of 3.5 (Waldmann R. et al., *J Biol Chem* 1997; **272**: 20975-8).

Tissue distribution of cloned proton-gated ion channels

ASIC1A and ASIC2B mRNAs are present in both brain and sensory neurons. ASIC2A mRNA is detected in the central nervous system but is absent in sensory neurons. ASIC1B and DRASIC are exclusively expressed in sensory neurons, predominantly in small diameter neurons. The different ASIC subunits in brain (ASIC1A, ASIC2A, ASIC2B) as well as the ASICs in sensory neurons (ASIC1B, ASIC2B and DRASIC) appear to be coexpressed in the same neurons (Waldmann R. et al., *Curr Opinion Neurobiol* 1998; **8**: 418-24).

Homo- and heteromultimeric assembly of proton-gated channel subunits

Ion channels are multimeric complexes, which result from the association of several identical (homopolymeric) and/or different (heteropolymeric) channel subunits. Except for ASIC2B, all ASIC subunits associate into homomultimeric complexes and yield the functional channels described above. In addition, certain ASIC subunits have been shown to form functional heteromultimeric channels with distinctive properties. Indeed, ASIC2B, which by itself does not give rise to a proton-gated channel, modifies

the channel characteristics of ASIC2A and of DRASIC when coexpressed with either one or the other. The homomultimeric ASIC2A channel has a single exponential inactivation profile and is highly selective for sodium. When coexpressed with ASIC2B, the inactivation kinetics become biphasic with a slowly inactivating component that poorly discriminates between sodium (Na^+) and potassium (K^+) ions. Similarly, when DRASIC was coexpressed with ASIC2B, the sustained sodium-selective current of DRASIC became cation non-selective ($pNa^+=pK^+$) (Lingueglia E. et al., *J Biol Chem* 1997; **272**: 29778-83).

Proton-gated channels are related to degenerins and mechanosensation

As mentioned above, ASICs belong to the DEG/ENaC superfamily of receptors, which also includes epithelial sodium channels (ENaC) involved in sodium homeostasis, the FMRFamide peptide-activated channel FaNaC from *Helix aspersa,* involved in neurotransmission, the ATP-gated cation channels, also involved in neurotransmission, as well as the degenerins of *Caenorhabditis elegans,* involved in mechanotransduction. As mentioned above, involvement of proton-gated ion channels in nociception seems likely. However, since many of the ASIC subunits are also expressed elsewhere than in sensory neurons, other functions than nociception must also be considered. In addition to their noxious effects, protons might play important roles as neurotransmitters as implied by reports of neuronal activity in response to fluctuations of pH. However, it still remains to be proven that sufficient pH changes can occur to activate ASIC channels with low pH_{50}, such as ASIC2A or ASIC2A+ASIC2B channels. Alternatively, ASIC channels might also be activated by other ligands, such as neuropeptides and neurotransmitters, or by mechanical energy, as suggested by their sequence homology with the other members of the DEG/ENaC superfamily.

SUMMARY OF INVENTION

The object of the present invention is to provide the primary structure, functional characterization and tissue distribution of a human non-inactivating amiloride-sensitive proton-gated ion channel, designed herein as hASIC3.

hASIC3 is not the orthologue of rat DRASIC as supported by the following evidence:

1) hASIC3 has 83% sequence identity with rat DRASIC while human and rat ASIC1A orthologues show an identity of 97.7% and human and rat ASIC2A orthologues show an identity of 99%. (Garcia-Anoveros J. et al., *Proc Natl Acad Sci* 1997; **94**: 1459-64).

2) Tissue distribution of hASIC3 is widespread throughout the body while rat DRASIC mRNA is restricted to sensory neurons (compare Fig. 6 and Waldmann R. et al., *J Biol Chem* 1997; **272**: 20975-8)

3) hASIC3 and rat DRASIC biphasic currents display different properties. In the following discussion «fast component» will refer to the rapidly inactivating current and «slow component » will refer to the sustained current, which follows the fast component:

- The fast and slow component of hASIC3 have similar pH_{50}s (3.66 and 3.82, respectively), while rat DRASIC has different pH_{50}s for the fast and slow components (6.5 versus 3.5, respectively) (compare Fig. 3 and Waldmann et al., *J Biol Chem* 1997; **272**: 20975-8).
- The slow component of hASIC3 is inhibited by amiloride while the slow component of rat DRASIC is potentiated by amiloride (compare Fig. 5A and 5B and Waldmann et al., J Biol Chem 1997; 272: 20975-8).
- Reversal potentials for the fast and slow components of hASIC3 differed by 15 mV (E_{rev} fast= +33 mV and E_{rev} slow = +48 mV), while both fast and slow components of rat DRASIC have the same E_{rev} of +32 mV. (compare Fig. 4 and Waldmann et al., *J Biol Chem* 1997; **272**: 20975-8).

Another aim of the present invention is to provide a DNA sequence encoding a novel subtype of a human non-inactivating amiloride-sensitive proton-gated ion channel, hASIC3 and derivatives thereof.

In accordance with the present invention there is provided an isolated nucleic acid molecule, which consists essentially of the nucleotide sequence depicted in SEQ ID No. 1, and derivatives thereof.

The isolated nucleic acid molecule of the present invention encodes a peptide consisting essentially of the amino acid sequence listed in SEQ ID No.: 2, and derivatives thereof.

RNA encoding the hASIC3 receptor, transcribable from DNA in accordance with the present invention (SEQ ID No.: 1) and substantially free from other RNAs, also forms part of the invention, and may be useful for a number of purposes including hybridization studies, *in vitro* translation as well as translation in appropriate *in vivo* systems such as *Xenopus* oocytes.

The present invention also relates to complete and/or partial complementary and/or antisense nucleotide sequences corresponding to the nucleotide sequence listed in SEQ ID No. 1.

In accordance with the present invention, there is provided a vector, preferably an expression vector, selected from the group consisting of plasmid, phage, retrovirus, baculovirus, adenovirus and integration elements, which include the isolated nucleic acid molecule of the present invention.

The present invention also relates to host cells transformed or transfected with a vector as described above. Host cells may be prokaryotic or eukaryotic and include mammalian cells (such as COS, CHO cells and human embryonic kidney cells, HEK293), insect cells, yeasts (such as *Saccharomyces cerevisiae*) and bacteria (such

as *Escherichia coli*). Host cells will either transiently express hASIC3 and/or derivatives thereof, as in the case of COS cells, or be stably transfected with a vector carrying the hASIC3 sequence and/or derivatives thereof. A CHO cell line or any other cell line that stably expresses hASIC3 and/or any derivatives thereof can be used for electrophysiological, calcium-influx, ion-imaging, ligand-binding, affinity purification, immunoprecipitation, western blotting and immunoblotting studies. Host cells which do not express the receptor may still be useful as cloning hosts.

Another object of the present invention is to provide a method to isolate a ligand that will bind the hASIC3 protein, or any derivative thereof.

A hASIC3 and/or a derivative thereof prepared by recombinant DNA technology in accordance with the invention has a number of uses, either *in situ* in the membrane of the expression host or in *in vitro* systems. In particular, the receptor can used as a screen for ligands and/or compounds useful in a variety of human (or other animal) diseases and conditions, such as pain, inflammation, ischemia and neurodegenerative disorders. Ligands refers to any chemical or biological entity that binds to any intracellular and/or extracellular region or portion of the hASIC3 and/or its derivatives. Such ligands include compounds present in combinatorial chemical libraries, peptide phage display libraries, extracts containing unknown compounds (for example plant extracts, marine life extracts, toxins, venoms), as well as biological molecules such as polyclonal and/or monoclonal antibodies, neurotransmitters, peptides or inorganic ions and metals.

For example, in accordance with the present invention there is provided a method of using the isolated nucleic acid molecule listed in SEQ ID No. 1, or a sequence which hybridizes under stringent conditions to the sequence listed in SEQ ID No. 1, to produce a peptide consisting essentially of the amino acid sequence listed in SEQ ID No. 2, which comprises the steps of:

a) transforming a host with a DNA sequence capable of encoding the peptide
b) incubating the host under conditions which allow the protein sequence to be expressed and
c) isolating by all means the protein from the host.

Recording or imaging the activity of the protein from the host can be performed to confirm or monitor the activity of the protein. This method can be reiterated, this time, with two or more DNA sequences encoding two or more different proteins, which results in the obtention of heteropolymeric channels.

Derivatives of hASIC3 include functional and/or structural variants as described below:

- Derivatives of hASIC3 include molecules whose sequence differs from the hASIC3 sequence by a modification and/or substitution and/or insertion and/or deletion of one or several amino acid residues as long as this modification

and/or substitution and/or insertion and/or deletion does not modify the functional and/or structural properties of the hASIC3 channel, mainly the activation by protons. The amino acid sequence of derivatives must be at least 85% or greater identical to the amino acid sequence of hASIC3. Such derivatives can be synthetized and/or analysed by a person skilled in the art following established techniques.

- Derivatives of hASIC3 also include molecules whose sequence differs from the hASIC3 sequence by a modification and/or substitution and/or insertion and/or deletion of one or several amino acid residues even if this modification and/or substitution and/or insertion and/or deletion does modify the functional and/or structural properties of the hASIC3 channel, rendering it non-or differently responsive to protons.

Examples of derivatives are hASIC3-like channel subunits corresponding to the partial nucleotide sequences (Expressed Sequence Tag available from public dbest databases, such as EST # AI024055, AA628357, AA448259, AA449322, hASIC3 channel subunits epitope-tagged on N-terminus and/or C-terminus, as well as hASIC3 channel subunit where the first 150-200 amino acids were substituted by a new and different amino acid sequence, in a similar fashion as reported for ASIC1A and ASIC1B, and ASIC2A and ASIC2B.

The present invention relates to the association of hASIC3 and derivatives thereof with other channel subunits such as the proton-gated ion channel subunits and derivatives thereof (for example ASIC1A, ASIC1B, ASIC2A, ASIC2B), or other channel subunits related to the DEG/ENaC superfamily of receptors, such as alpha-, beta-, gamma-,delta-ENaC subunits or the P2x ATP-gated ion channel subunits. Such associations include interactions between subunits of different species, but the preferred associations are between subunits from the same species.

An example of heteromeric association between hASIC3 and rat P2x2 is given herein below. The resulting new channel is a novel proton-gated ion channel, which displays higher sensitivity to pH. Indeed, when hASIC3 and P2x2 are coinjected into *Xenopus* oocytes, inward currents can be activated with slight acidification to pH 6.5, while the homomeric hASIC3 requires a pH of 4.0. This different pharmacological property proves that hASIC3 and P2x2 physically associated to generate a new ion channel.

DESCRIPTION OF INVENTION

This invention will be described by way of specific embodiments, examples and figures, which purpose is to illustrate the invention rather than to limit its scope.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Nucleotide and predicted amino acid sequence of hASIC3 cDNA (SEQ ID No. 1 and No. 2, respectively) (A), deposited in Genbank database under the accession number AF057711. Two stretches of 20-34 amino acids corresponding to potential transmembrane domains, identified in Kyte-Doolitle hydrophobicity plot, are highlighted. Consensus phosphorylation sites in intracellular domains and N-glycosylation sites in extracellular domain are indicated by circled and boxed residues, respectively. Homology (in %) of hASIC3 (B) and phylogenetic relationships (C) with known members of the human ASIC/ENaC family. Protein dendrogram generated using UPGMA algorithm (Geneworks 2.5.1, Oxford Molecular Group).

Figure 2. Biphasic current phenotype of homomeric hASIC3 channels: effects of increasing rates of pH change. Oocytes were clamped at -70 mV and continuously perfused with Ringer's buffer containing 10 mM HEPES at pH 7.6. pH was then dropped to 4.0 for 10 sec with increasing pH gradients obtained by raising the buffer capacity differential between control and test buffers: A. pH 7.6 to pH 4.0 in 10 mM HEPES; B. pH 7.6 in 5 mM to pH 4.0 in 10 nM HEPES; and C. pH 7.6 in 5 mM to pH 4.0 in 20 mM HEPES. Controls done with the test buffers at pH 7.6 did not activate the hASIC3 channel. Oocytes injected with injection buffer alone showed no inward currents. The amplitude of the early but not the late component, and thus the ratio of early/late peak currents, appears to be very sensitive to the speed at which the pH drops from normal to acid pH values.

Figure 3. Dose-response curves of pH activation of hASIC3 currents. Dose-response curves were constructed in 20 mM HEPES buffered at different pH values from 6.0 to 3.0. Peak currents of fast and slow currents were analysed with the four parameter logistic equation and the partial F test for statistical comparison. Each point represents mean Ò SEM from this typical experiment. Apart from the maximal response, no other significant difference was observed between both curves.

Figure 4. Current-voltage (I/V) relationship of the fast and slow hASIC3 currents. Recordings were done in Ringer's buffer containing (in mM): NaCl 115, KCl 2.5, $CaCl_2$ 1.8 and HEPES 5, pH 7.6. The I/V relationship was established by measuring peak currents of both fast and slow responses to pH 4.0 (applied 1 sec after voltage step) at different membrane potentials, after substracting background currents recorded without pH applications (A). Peak current values were plotted (B) and reversal potential estimated from linear (slow current) and nonlinear (fast current) regression analysis. Panels A and B represent a typical experiment where reversal potentials were 33.4 mv and 44.8 mv respectively for the fast and slow currents.

Figure 5. Differential sensitivity of the fast and slow hASIC3 currents to amiloride. hASIC3 currents were activated by pH 4.0 in the presence and absence of 100 ÊM amiloride (A). Inhibition by amiloride was much stronger on the fast than the sustained current. Data in B represent mean Ò SEM (n= 17) of residual peak currents during

amiloride application for the fast (37.2 Ò 6.4%) and sustained (72.0 Ò 3.8%) components expressed as percent of controls. Statistical significance (P) was evaluated by unpaired two-tailed t-test (*** P<0.001).

Figure 6. Distribution of hASIC3 mRNA in normal human tissues. High stringency hybridization of radiolabeled hASIC3 cDNA on human polyA+ RNA isolated from brain, spinal cord, internal tissues as well as from 17-28 weeks fetal tissues was quantitated by densitometric analysis in phosphorimaging. Amounts of polyA+ RNA target were normalized across tissues to allow direct comparisons of transcription levels (see Materials and Methods for details).

Figure 7. High levels of transcription of hASIC3 gene in sensory ganglia enriched in nociceptive neurons. Localization of hASIC3 and reporter G3PDH mRNAs in human trigeminal ganglia (TG), cerebellum (CB) and lung (L) using RT-PCR amplification with specific exact match primers (see Materials and Methods section for details).

Figure 8. Illustration of a recording of non-inactivating cation current induced by strong acid (pH 4.0) in *Xenopus* oocytes injected with hASIC3 clone alone in pcDNA3 vector.

Figure 9. Illustration of a recording of non-inactivating cation current induced by weak acid (pH 6.5) in *Xenopus* oocytes coinjected with hASIC3 clone and rat P2x2 clone, both in pcDNA3 vector.

EXAMPLE 1: Characterization of a human non-inactivating proton-gated ion channel, hASIC3

Introduction

Acid sensing is a specific kind of chemoreception that plays a critical role in the detection of nociceptive pH imbalances occurring in conditions of cramps, trauma, inflammation and hypoxia (Lindahl, 1974). In mammals, a population of small-diameter primary sensory neurons in the dorsal root ganglia and trigeminal ganglia express specialized pH-sensitive surface receptors activated by increase of extracellular proton concentration (Bevan and Yeats, 1991). Native electrophysiological responses of sensory neurons to applications of pH 5.8-6.5 are characterized by a fast desensitizing inward current followed by a slow sustained current (Krishtal and Pidoplichko, 1981). Elucidating the native molecular composition of proton sensors in human sensory neurons will be an important step in the rational development of a novel class of analgesics. A family of genes coding for neuronal proton-gated channels subunits has been recently discovered (Garcia-Anoveros et al., 1997; Waldmann et al., 1997). Heterologously expressed amiloride-sensitive homomeric rat ASIC (Acid Sensing Ion Channel) (Waldmann et al., 1997) responds to small pH changes by a fast desensitizing sodium-selective current, while MDEG1 (Mammalian DEGenerin 1) (Waldmann et al., 1996) and DRASIC (Dorsal Root ganglia ASIC) (Waldmann et al., 1997) require drastic pH changes to gate desensitizing and biphasic currents, respectively. ASIC and MDEG1 can associate together to generate a heteromeric

channel activated at low pH (<5) with unique kinetics and ionic selectivities (Bassilana et al., 1997). A neuronal splicing variant of MDEG1 was shown to modulate DRASIC biophysical properties by heteromeric association (Lingueglia et al., 1997). These proton-gated channels share a putative two-transmembrane domain topology and co-localization in small diameter capsaicin-sensitive sensory neurons with P2X ATP-gated channels (North, 1997). From their sequence, they belong to an expanding gene superfamily including mammalian epithelial sodium channels (Canessa et al., 1994), pickpocket (PPK) and ripped pocket (RPK) subunits from *Drosophila* (Adams et al., 1998), degenerins of *C. elegans* (Corey and Garcia-Anoveros, 1996), and the FMRFamide-gated channel of *Helix aspersa* (Lingueglia et al., 1995). Despite their potential importance in monitoring pH changes in central nervous system and sensory pathways, human proton receptor genes have not yet been functionally characterized. We report here for the first time the heterologous expression of a human proton-gated channel, as well as significant inter-species differences observed both in functional properties and regional distributions of acid sensors.

Material and Methods

Molecular cloning

Using the tblastn algorithm, virtual screening of the dbEST database of NCBI (Lennon et al., 1996) with probes corresponding to the protein motif LXFPAVTLCNXNXXRXS, conserved in all known members of the degenerin/ENaC/ASIC family, led to the identification of human EST sequences encoding a novel member of the proton sensor gene family (Genbank accession numbers AA449579 and AA429417). The clone tagged by 5' EST AA449579 and by 3' EST AA449322 from a total fetus cDNA library was sequenced on both strands using walking primers and an ALF DNA sequencer (Pharmacia-LKB). Full-length hASIC3 was directionally subcloned into unique EcoRI and NotI sites of eukaryotic vector pcDNA3 (Invitrogen) for CMV promoter-driven heterologous expression in *Xenopus* oocytes.

Electrophysiology in *Xenopus* oocytes

Oocytes surgically removed from adult *Xenopus* laevis were treated for 2 h at room temperature with type II collagenase (Gibco-BRL) in Barth's solution under constant agitation. Selected oocytes at stage IV-V were defolliculated manually before nuclear micro-injections (Bertrand et al., 1991) of 5 ng of hASIC3 in pcDNA3 vector. After 2-4 days of expression at 19°C in Barth's solution containing 50µg/ml gentamycin, currents were recorded in the two-electrode voltage clamp configuration using an OC-725B amplifier (Warner Instruments). Whole cell currents were acquired and digitized at 500 Hz on a Macintosh IIci computer with an A/D NB-MIO16XL interface (National Instruments) then recorded traces were post-filtered at 100 Hz in Axograph (Axon Instruments). Agonist, amiloride and wash solutions were prepared in a modified

Ringer's solution containing 115 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$ in 5-20 mM HEPES (Sigma) buffer adjusted with NaOH or HCl at pH 2 to 8 and applied on oocytes by constant perfusion (10-12 ml/min) at room temperature. Mean values ± SEM corresponded to measurements from a minimum of 5 oocytes.

RT-PCR and mRNA dot blot hybridization

Total RNA from post-mortem samples of normal human trigeminal ganglia were isolated using Trizol reagent (Gibco-BRL), then 1 Êg was subjected to random-primed reverse transcription using Superscript (Gibco-BRL). Around 100 ng of RT-cDNA was used as template for PCR with Expand DNA polymerase (Boerhinger-Mannheim). Specific hASIC3 primers TCAGTGGCCACCTTCCTCTA (forward) and ACAGTCCAGCAGCATGTCATC (reverse) were used to amplify the region corresponding to nucleotides 175-513 (Fig.IA). After initial template denaturation of 2 min at 94-C, thermal cycles consisted of 45 sec at 94°C, 45 sec at 55°C and 2 min at 72°C for 30 cycles. Molecular identity and homogeneity of PCR products were checked by sizing and specific restriction patterns. Initial sample loading was checked by co-amplification of glyceraldehyde 3-phosphate dehydrogenase (G3PDH) housekeeping mRNA. RNA samples not subjected to reverse transcription but PCR amplified in identical conditions provided our negative controls.

Known amounts of human polyA+ RNA (89-514 ng), isolated from various fetal and adult normal tissues and normalized for the transcription levels of several housekeeping genes (Clontech), were dot-blotted and probed with the [32P]-labeled EcoRI-XbaI fragment of hASIC3 cDNA at high stringency (final elution at 65°C in 0.3 X SSC buffer for 10 min). After exposure for 16 hours, hybridization signals were acquired and quantitated using a Storm phosphorimager (Molecular Dynamics), then analyzed in densitometry with ImageQuant software (Molecular Dynamics).

Results

Primary structure of hASIC3 channel subunit

Sequence analysis of the 1.7 kb-long hASIC3 polyA+ mRNA revealed an open reading frame encoding 531 amino acids (Fig. IA), with initiation of translation at the proximal Met codon located at position nt. 22. The predicted molecular weight of 59 kDa for the immature protein was confirmed by *in vitro* translation (data not shown). According to the current topological model based on primary structure analysis and biochemical tests, a large domain of 365 amino acids faces the extracellular side of the plasma membrane (Canessa et al., 1994). In this extracellular domain, a total of 15 cysteine residues are highly conserved in the ASIC family, with the exception of Cys267 being absent in human BNaC1 (hASIC2) only. Two potential sites for Asn-linked glycosylation, Asn175 and Asn398, are located in this Cysteine-rich loop. Consensus sites for phosphorylation by casein kinase II (Ser5) and by protein kinase C (Ser39, Ser478, Ser493, Ser521) are found in the intracellular N-terminal domain of

hASIC3 as well as in the intracellular C-terminal domain (Fig. lA). The hASIC3 subunit displays 83% of identity with rat DRASIC subunit at the amino acid level, 48% with human BNaC2 (hASIC1) and 47% with BNaC1 (hASIC2) (Fig. 1B). Therefore hASIC3 belongs to the proton-gated channel family, itself a branch of the degenerin/ENaC/FMRFamide-gated channel phylogenetic tree (Fig. 1C).

Functional and pharmacological properties of homomeric hASIC3 channels

When heterogously expressed in *Xenopus* oocytes, hASIC3 subunits assemble into functional homomeric channels activated by low extracellular pH (Fig. 2A). Rapid changes of extracellular pH (Fig. 2B-C) revealed a biphasic response. This unique phenotype was characterized by a fast and rapidly desensitizing current followed by a slow and sustained current which returned to baseline only upon return to physiological pH. The relative amplitude of the fast current appeared dependent on the slope of the pH gradient applied (Fig. 2A-C). However, we found the pH sensitivity of the two hASIC3-mediated currents to be almost identical with a pH50 of 3.66 Ò 0.06 (fast) vs 3.82 Ò 0.04 (slow) (Fig. 3). The positive cooperativity reflected in the dose-response curve profile, nHfast=1 .57 Ò 0.3 and nHslow=1 .55 Ò 0. 17, indicated that at least two protonations on two subunits are required in order to gate the cation channel. To study possible differences of ionic selectivity between fast and slow components, we performed a current-voltage relationship at pH 4.0 in normal Ringer. The peak amplitude of the fast component displayed some voltage-dependence from its slight inward rectification, while the slow and sustained component was ohmic in the range from -70 to + 70 mv (Fig. 4B). Furthermore, although both reversal potentials were greater than 30 mv as expected for channels conducting mainly sodium, we measured a _Erev= +15 Ò 3.2 mv ($p < 0.01$) between the fast (+32.9 Ò 4.4 mv) and the slow component (+48.2 Ò 4.8 mv) (Fig. 4A-B). These two phases of proton-induced hASIC3 current differed also by their sensitivity to the antagonist amiloride. Co-application of 100 ÊM amiloride with pH 4.0 in conditions of biphasic response demonstrated a more efficient blockade of the fast (62.8 Ò 6.5%) than of the slow current (28.7 Ò 4.6%) by amiloride (Fig. SAB).

Central and peripheral distribution of hASIC3 gene expression

As a rough index of anatomical distribution and mRNA abundance, we noticed several cDNAs encoding hASIC3 in total fetus and testis cDNA libraries representated in the dbEST database. Results obtained in RNA hybridization at high stringency confirmed that the hASIC3 gene is transcribed in a wide spectrum of internal organs as well as in the central nervous system (Fig. 6). In the adult stage, hASIC3 transcripts were detected in lung, lymph nodes, kidney, pituitary, heart and testis as well as in brain and spinal cord. A developmental up-regulation of hASIC3 gene expression was apparent when comparing fetal vs adult mRNA levels in lung and kidney (Fig. 6). Thus hASIC3 subunit expression is not restricted exclusively to sensory ganglia as is rat

DRASIC, explaining our decision to use a chronological nomenclature that is distribution-independent. The potentially important function of non-inactivating proton-gated channels in nociceptive sensory neurons was nevertheless confirmed by the detection of high levels of ASIC3 mRNA in adult human trigeminal ganglia using RT-PCR (Fig. 7).

EXAMPLE 2: Heteromultimeric channel composed of hASIC3 and rat P2x2

To demonstrate the possibility of heteromultimeric association between hASIC3 and/or derivatives thereof and other ion channel subunits, we have coexpressed the hASIC3 and rat P2x2 constructs in *Xenopus* oocytes to verify if the presence of an ATP-gated ion channel subunit can modify hASIC3 channel properties. P2X2 displays important similarities with hASIC3, such as overall membrane topology (two transmembrane spanning domains, cysteine rich extracellular domain), sensitivity to pH (response to ATP of the P2X2 homomultimeric channel is strongly potentiated by acid pH), and tissue distribution (both hASIC3 and P2X2 are colocalized in sensory neurons).

There is shown in Fig. 8 the recording of non-inactivating cationic current induced by strong acid (pH 4.0) in *Xenopus* oocytes injected with the hASIC3 clone alone in pcDNA3 vector. These data demonstrate that hASIC3 alone can associate in functional homomeric cation channels.

There is shown in Fig. 9 the recording of non-inactivating cation current induced by weak acid (pH 6.5) in *Xenopus* oocytes coinjected with hASIC3 clone and rat P2x2 clone, both in pcDNA3 vector. These data demonstrate that the co-expression of hASIC3 and rat P2x2 changes the pH sensitivity of homomeric hASIC3 or leads to the formation of heteromultimeric pH-sensitive channels.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

References

Adams C.M., Anderson M.G., Motto D.G., Price M.P., Johnson W.A., and Welsh M.J. (1998) Ripped pocket and pickpocket, novel Drosophila DEG/ENaC subunits expressed in early development and in mechanosensory neurons. J. Cell Biol. 140, 143-152.

Bassilana F., Champigny G., Waldmann R., de Weille J.R., Heurteaux C., and Lazdunski M. (1997) The acid-sensitive ionic channel subunit ASIC and the

mammalian degenerin MDEG form a heteromultimeric H+-gated Na+ channel with novel properties. J. Biol. Chem. 272, 28819-28822.

Bertrand D., Cooper E., Valera S., Rungger D., and Ballivet M. (1991) Electrophysiology of neuronal nicotinic acetylcholine receptors expressed in Xenopus oocytes following nuclear injection of genes or cDNAs. In: Methods in Neurosciences (Conn M.P., ed.) pp 174-193. New York: Academic.

Bevan S ., and Winter J. (1995) Nerve growth factor (NGF) differentially regulates the chemosensitivity of adult rat cultured sensory neurons. J. Neurosci. 15, 4918-4926.

Bevan S., and Yeats J. (1991) Protons activate a cation conductance in a sub-population of rat dorsal root ganglion neurones. J. Physiol. 433, 145-161.

Canessa C.M., Merillat A.M., and Rossier B.C. (1994) Membrane topology of the epithelial sodium channel in intact cells. Am. J. Physiol. 267, C1682-1690,

Canessa C.M., Schild L., Buell G., Thorens B., Gautschi I., Horisberger J.-D., and Rossier B.C. (1994) Amiloride-sensitive epithelial Na+ channel is made of three homologous subunits. Nature 367, 463-467.

Corey D.P., and Garcia-Anoveros J. (1996) Mechanosensation and the DEG/ENaC ion channels. Science 273, 323-324.

Coscoy S., Lingueglia E., Lædunski M., and Barbry P. (1998) The Phe-Met-Arg-Phe-amide-activated sodium channel is a tetramer. J. Biol. Chem. 273, 8317-8322.

De LÇan A., Munson P.J., and Rodbard D. (1978) Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. Am. J. Physiol. 235, E97-E102.

Dray A., and Perkins M. (1993) Bradykinin and inflammatory pain. Trends Neurosci. 16, 99-104.

Firsov D., Gautschi I., Merillat A.-M., Rossier B.C., and Schild L. (1998) The heterotetrameric architecture of the epithelial sodium channel (ENaC). The EMBO J. 17, 344-352.

Garcia-Anoveros J., Derfler B., Neville-Golden J., Hyman B.T., and Corey D.P. (1997) BNaCl and BNaC2 constitute a new family of human neuronal sodium channels related to degenerins and epithelial sodium channels. Proc. Natl. Acad. Sci. USA 94, 1459-1464.

Ishibashi K., and Marumo F. (1998) Molecular cloning of a DEG/ENaC sodium channel from human testis. Biochem. Biophys. Res. Comm. 245, 589-593.

Kleyman T.R., and Cragoe E.J. (1988) Amiloride and its analogs as tools in the study of ion transport. J. Membr. Biol. 105, 1-21.

Konnerth A., Lux H.D., and Morad M. (1987) Proton-induced transformation of calcium channel in chick dorsal root ganglion cells. J. Physiol. 386, 603-33.

Krishtal O.A., and Pidoplichko V.I. (1981) A receptor for protons in the membrane of sensory neurons may participate in nociception. Neuroscience 6, 2599-2601.

Krishtal O.A., Osipchuk Y.V., Shelest T.N., and Smirnoff S.V. (1987) Rapid extracellular pH transients related to synaptic transmission in rat hippocampal slices. Brain Res. 436, 352356.

Lennon G., Auffray C., Polymeropoulos M., and Soares M.B. (1996) The I.M.A.G.E. consortium; an integrated molecular analysis of genomes and their expression. Genomics 33, 151-152.

Lindahl O. (1974) Pain- a general chemical explanation. Advances Neurol. 4, 45-47.

Lingueglia E., Champigny G., Lazdunski M., and Barbry P. (1995) Cloning of the amiloride-sensitive FNIRFamide peptide-gated sodium channel. Nature 378, 730-733.

Lingueglia E., de Weille J.R., Bassilana F., Heurteaux C., Sakai H., Waldmann R., and Lazdunski M. (1997) A modulatory subunit of acid sensing ion channels in brain and dorsal root ganglion cells. J. Biol. Chem. 272, 29778-29783.

North R.A. (1997) Families of ion channels with two hydrophobic segments. Current Opinion in Cell Biology 8, 474-483.

Price M.P., Snyder P.M., and Welsh M.J. (1996) Cloning and expression of a novel human brain Na+ channel. J. Biol. Chem. 271, 7879-7882.

Snyder P.M., Cheng C., Prince L.S., Rogers J.C., and Welsh MJ. (1998) Electrophysiological and biochemical evidence that DEG/ENaC cation channels are composed of nine subunits. J. Biol. Chem. 273, 681-4.

Tavernarakis N., Shreffler W., Wang S., and Driscoll M. (1997) unc-8, a DEG/ENaC family member, encodes a subunit of a candidate mechanically gated channel that modulates C. elegans locomotion. Neuron 18, 107-119.

Waldmann R., Bassilana F., de Weille J., Champigny G., Heurteaux C., and Lazdunski, M. (1997) Molecular cloning of a non-inactivating proton-gated Na+ channel specific for sensory neurons. J. Biol. Chem. 272, 20975-20978.

Waldmann R., Champigny G., Bassilana F., Heurteaux C., and Lazdunski M. (1997) A proton-gated cation channel involved in acid-sensing. Nature 386, 173-177.

Waldmann R., Champigny G., Voilley N., Lauritzen I., and Lazdunski M. (1996) The mammalian degenerin MDEG, an amiloride-sensitive cation channel activated by mutations causing neurodegeneration in Caenorhabditis elegans. J. Biol. Chem. 271, 0433-10436.

Claims:

1. The use of a molecule having the amino acid sequence of SEQ ID NO:1 or a variant having at least 85% identity therewith, as a proton-gated cation channel.

2. The use as defined in C1, wherein said molecule forms a homopolymeric channel.

3. The use as defined in C1, in combination with another cation channel sub-unit which forms a heteropolymeric channel with said molecule.

4. The use as defined in C3, wherein said other cation channel belongs to the degenerin/ENaC channel superfamily.

5. The use as defined in C3, wherein said other cation channel is a P2X ATP-gated channel.

6. The use as defined in C5, wherein said other cation channel sub-unit is P2X2 ATP-gated channel sub-unit.

7. A recombinant host cell comprising a first and a second DNA sequences respectively capable of expressing the molecule and the other cation channel as defined in any one of claims 3 to 6.

8. The use of the recombinant host cell as defined in C7, in the making of a composition or a kit for screening compounds useful as proton-gated cation channel ligands.

9. The use of the recombinant host cell as defined in C7 for screening compounds useful as proton-gated cation channel ligands.

10. The use of a nucleic acid encoding a molecule as defined in any one of C1 to 6, in the making of a composition or a kit for screening compounds useful as proton-gated cation channel ligands.

11. The use of a nucleic acid encoding a molecule as defined in any one of C1 to 6, for screening compounds useful as proton-gated cation channel ligands.

12. The use of a molecule as defined in any one of C1 to 6, in the making of a composition or a kit for screening compounds useful as proton-gated cation channel ligands.

13. The use of a molecule as defined in any one of C1 to 6, for screening compounds useful as proton-gated cation channel ligands.

14. A composition or kit for screening compounds useful as proton-gated cation channel ligands, which comprises a molecule as defined in any one of C1 to 6, a nucleic acid encoding said molecule or a recombinant host capable of expressing said molecule, and any suitable auxiliary component.

15. The use as defined in any one of C8 to 13, or the composition or kit as defined in C14, wherein said ligands are analgesics.

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TCGCACGACGCGGTTCTGGCCATGAAGCCCACCTCAGGCCCAGAGGAGGCCCGGCGGCAGCCCTCGGACATCCGC   75
                      M  K  P  T  S  G  P  E  E  A  R  R  Q  P  S  D  I  R     18
GTGTTCGCCAGCAACTGCTCGATGCACGGGCTGGGCCACGTCTTCGGGCCAGGCAGCCTGAGCCTGCGCCGGGGG  150
V  F  A  S  N  C  S  M  H  G  L  G  H  V  F  G  P  G  S  L  S  L  R  R  G     43
ATGTGGGCAGCGGCCGTGGTCCTGTCAGTGGCCACCTTCCTCTACCAGGTGGCTGAGAGGGTGCGCTACTACAGG  225
M  W  A  A  A  A  V  V  L  S  V  A  T  F  L  Y  Q  V  A  E  R  V  R  Y  Y  R     68
GAGTTCCACCACCAGACTGCCCTGGATGAGCGAGAAAGCCACCGGCTCGTCTTCCCGGCTGTCACCCTGTGCAAC  300
E  F  H  H  Q  T  A  L  D  E  R  E  S  H  R  L  V  F  P  A  V  T  L  C  N     93
ATCAACCCACTGCGCCGCTCGCGCCTAACGCCCAACGACCTGCACTGGGCTGGGTCTGCGCTGCTGGGCCTGGAT  375
I  N  P  L  R  R  S  R  L  T  P  N  D  L  H  W  A  G  S  A  L  L  G  L  D    118
CCCGCAGAGCACGCCGCCTTCCTGCGCGCCCTGGGCCGGCCCCCTGCACCGCCCGGCTTCATGCCCAGTCCCACC  450
P  A  E  H  A  A  F  L  R  A  L  G  R  P  P  A  P  P  G  F  M  P  S  P  T    143
TTTGACATGGCGCAACTCTATGCCCGTGCTGGGCACTCCCTGGATGACATGCTGCTGGACTGTCGCTTCCGTGGC  525
F  D  M  A  Q  L  Y  A  R  A  G  H  S  L  D  D  M  L  L  D  C  R  F  R  G    168
CAACCTTGTGGGCCTGAGAACTTCACCACGATCTTCACCCGGATGGGAAAGTGCTACACATTTAACTCTGGCGCT  600
Q  P  C  G  P  E  N  F  T  T  I  F  T  R  M  G  K  C  Y  T  F  N  S  G  A    193
GATGGGGCAGAGCTGCTCACCACTACTAGGGGTGGCATGGGCAATGGGCTGGACATCATGCTGGACGTGCAGCAG  675
D  G  A  E  L  L  T  T  T  R  G  G  M  G  N  G  L  D  I  M  L  D  V  Q  Q    218
GAGGAATATCTACCTGTGTGGAGGGACAATGAGGAGACCCCGTTTGAGGTGGGGATCCGAGTGCAGATCCACAGC  750
E  E  Y  L  P  V  W  R  D  N  E  E  T  P  F  E  V  G  I  R  V  Q  I  H  S    243
CAGGAGGAGCCGCCCATCATCGATCAGCTGGGCTTGGGGGTGTCCCCGGGCTACCAGACCTTTGTTTCTTGCCAG  825
Q  E  E  P  P  I  I  D  Q  L  G  L  G  V  S  P  G  Y  Q  T  F  V  S  C  Q    268
CAGCAGCAGCTGAGCTTCCTGCCACCGCCCTGGGGCGATTGCAGTTCAGCATCTCTGAACCCCAACTATGAGCCA  900
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Fig. 1A

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Q  Q  Q  L  S  F  L  P  P  P  W  G  D  C  S  S  A  S  L  N  P  N  Y  E  P    293
GAGCCCTCTGATCCCCTAGGCTCCCCCAGCCCCAGCCCCAGCCCTCCCTATACCCTTATGGGGTGTCGCCTGGCC  975
E  P  S  D  P  L  G  S  P  S  P  S  P  S  P  P  Y  T  L  M  G  C  R  L  A    318
TGCGAAACCCGCTACGTGGCTCGGAAGTGCGGCTGCCGAATGGTGTACATGCCAGGCGACGTGCCAGTGTGCAGC 1050
C  E  T  R  Y  V  A  R  K  C  G  C  R  M  V  Y  M  P  G  D  V  P  V  C  S    343
CCCCAGCAGTACAAGAACTGTGCCCACCCGGCCATAGATGCCATCCTTCGCAAGGACTCGTGCGCCTGCCCCAAC 1125
P  Q  Q  Y  K  N  C  A  H  P  A  I  D  A  I  L  R  K  D  S  C  A  C  P  N    368
CCGTGCGCCAGCACGCGCTACGCCAAGGAGCTCTCCATGGTGCGGATCCCGAGCCGCGCCGCCGCGCGCTTCCTG 1200
P  C  A  S  T  R  Y  A  K  E  L  S  M  V  R  I  P  S  R  A  A  A  R  F  L    393
GCCCGGAAGCTCAACCGCAGCGAGGCCTACATCGCGGAGAACGTGCTGGCCCTGGACATCTTCTTTGAGGCCCTC 1275
A  R  K  L  [N] R  S  E  A  Y  I  A  E  N  V  L  A  L  D  I  F  F  E  A  L    418
AACTATGAGACCGTGGAGCAGAAGAAGGCCTATGAGATGTCAGAGCTGCTTGGTGACATTGGGGGCCAGATGGGC 1350
N  Y  E  T  V  E  Q  K  K  A  Y  E  M  S  E  L  L  G  D  I  G  G  Q  M  G    443
CTTTTCATCGGGGCCAGCCTGCTCACCATCCTCGAGATCCTAGACTACCTCTGTGAGGTGTTCCGAGACAAGGTC 1425
L  F  I  G  A  S  L  L  T  I  L  E  I  L  D  Y  L  C  E  V  F  R  D  K  V    468
CTGGGATATTTCTGGAACCGACAGCACTCCCAAAGGCACTCCAGCACCAATCTGCTTCAGGAAGGGCTGGGCAGC 1500
L  G  Y  F  W  N  R  Q  H  (S) Q  R  H  S  S  T  N  L  L  Q  E  G  L  G  (S)  493
CATCGAACCCAAGTTCCCCACCTCAGCCTGGGCCCCAGACCTCCCACCCCTCCCTGTGCCGTCACCAAGACTCTC 1575
H  R  T  Q  V  P  H  L  S  L  G  P  R  P  P  T  P  P  C  A  V  T  K  T  L    518
TCCGCCTCCCACCGCACCTGCTACCTTGTCACACAGCTCTAGACCTGCTGTCTGTGTCCTCGGAGCCCCGCCCTG 1650
S  A  (S) H  R  T  C  Y  L  V  T  Q  L  .                                     531
ACATCCTGGACATGCCTAGCCTGCACGTAGCTTTTCCGTCTTCACCCCAAATAAAGTCCTAATGCATCAAAAAAA 1725
AAAAAAA                                                                       1732
```

Fig - 1B

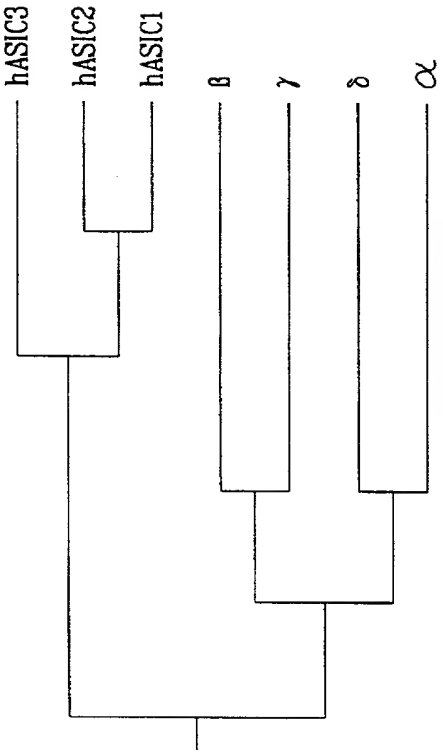

FIG - 1D

	hASIC2	hASIC1	α	β	γ	δ
hASIC3	47.9	46.6	17.3	15.4	16.6	14.9
hASIC2		64.5	15.2	17.2	16.4	15.0
hASIC1			13.1	17.0	16.9	15.0
α				26.7	29.1	33.4
β					31.4	22.6
γ						21.8

FIG - 1C

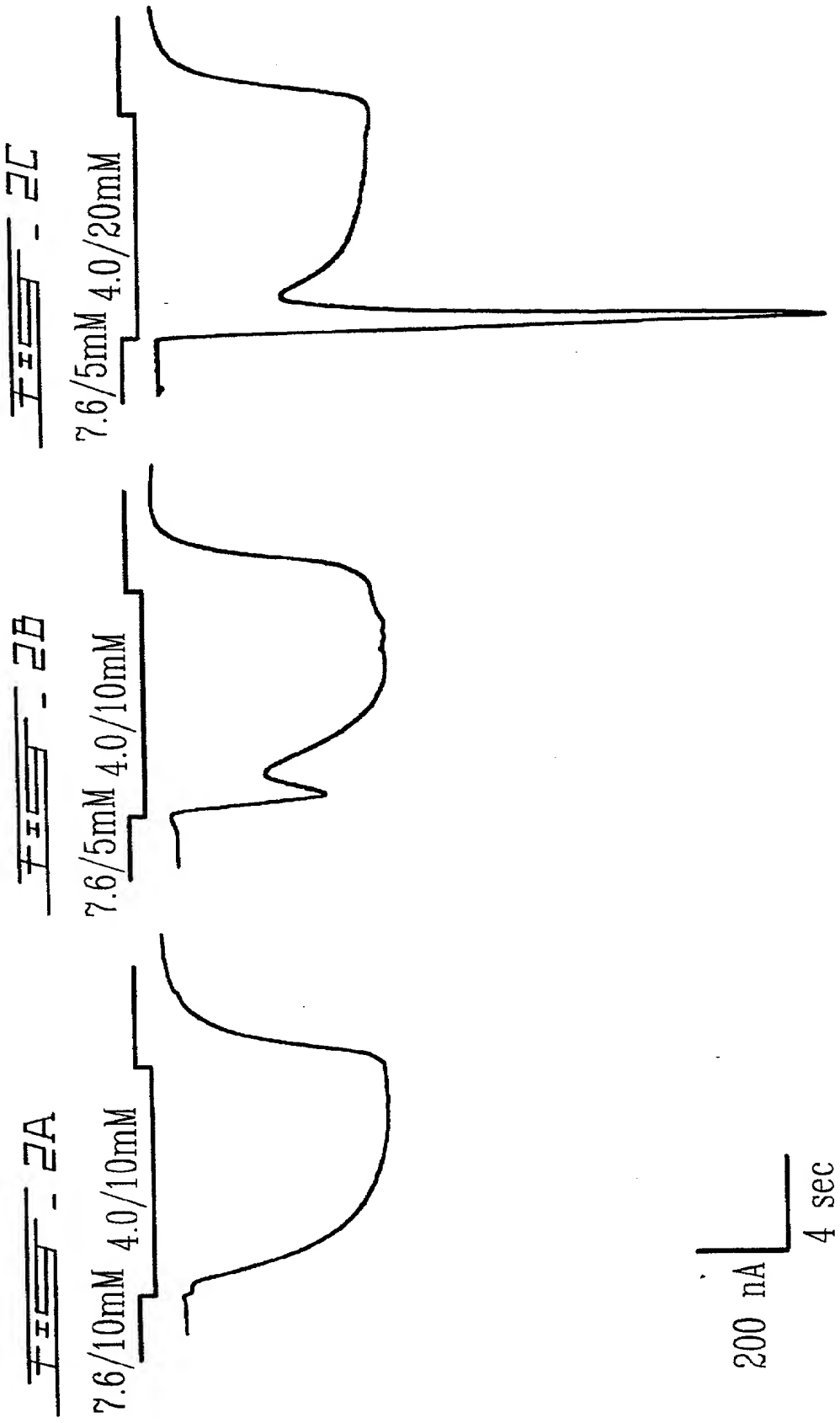
FIG - 2A
7.6/10mM 4.0/10mM
FIG - 2B
7.6/5mM 4.0/10mM
FIG - 2C
7.6/5mM 4.0/20mM
200 nA
4 sec

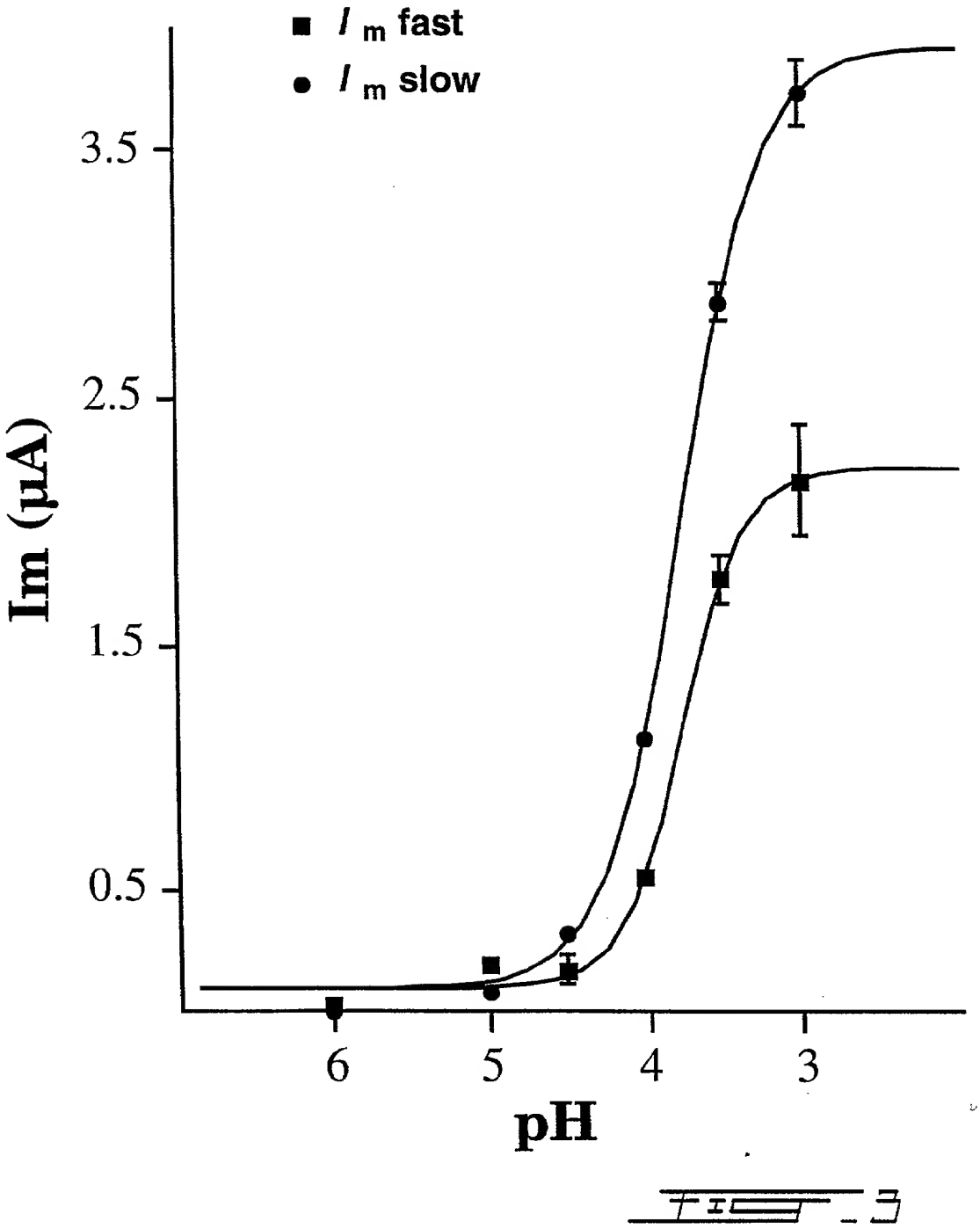

Fig. 3

6/11

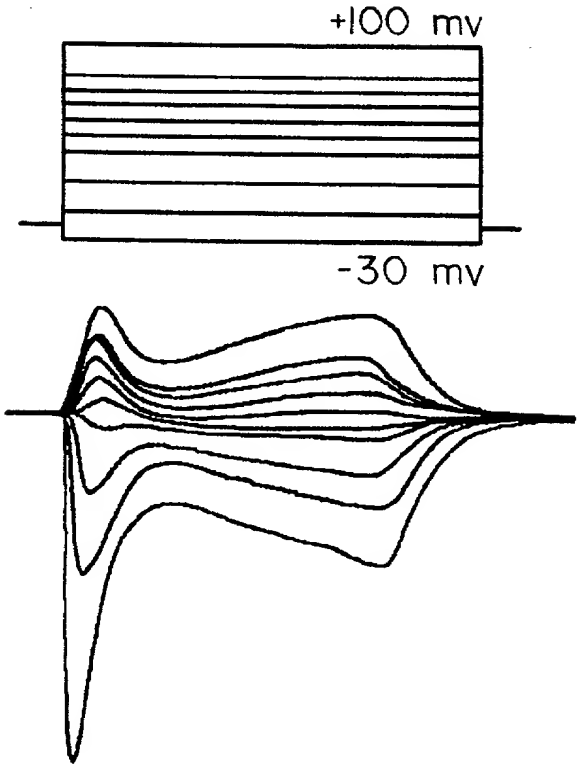

Fig. 4A

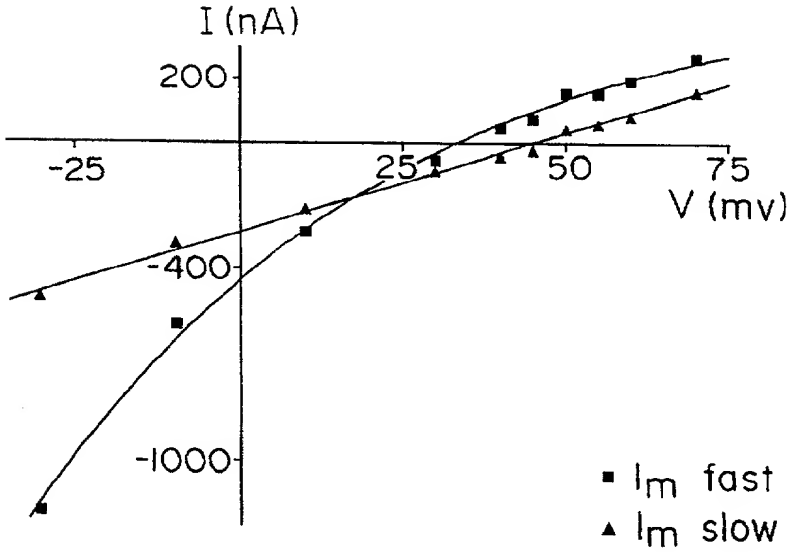

Fig. 4B

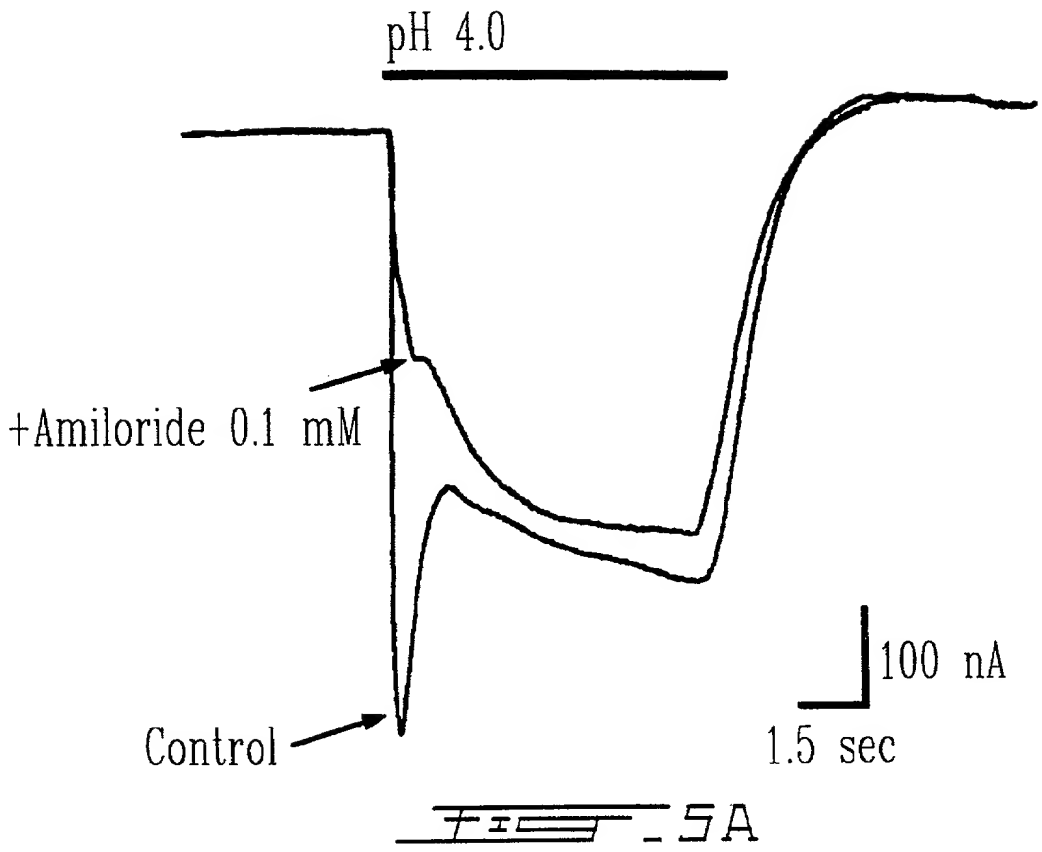
pH 4.0
+Amiloride 0.1 mM
Control
100 nA
1.5 sec

FIG. 5A

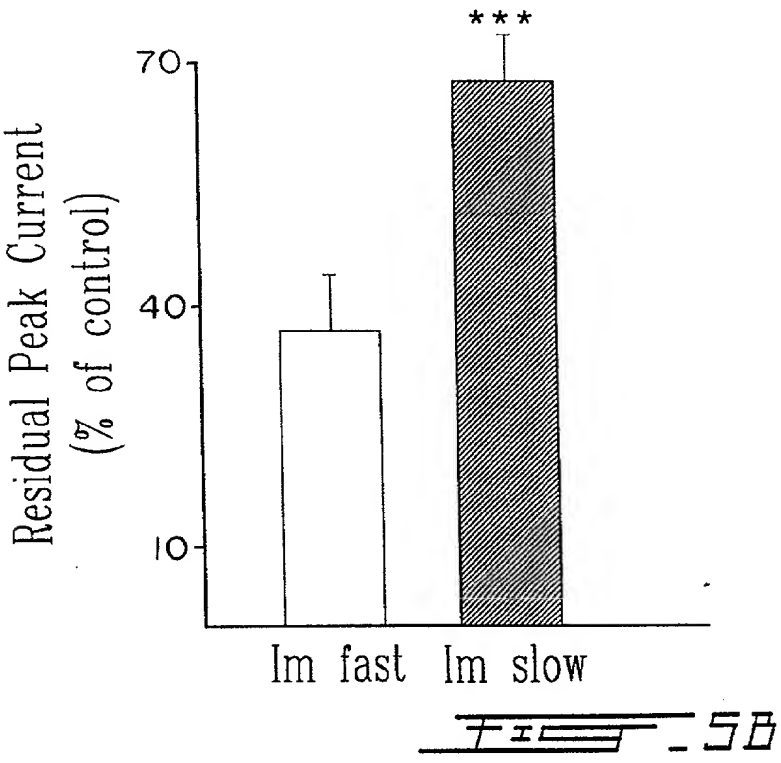
***
70
40
10
Residual Peak Current
(% of control)
Im fast
Im slow

FIG. 5B

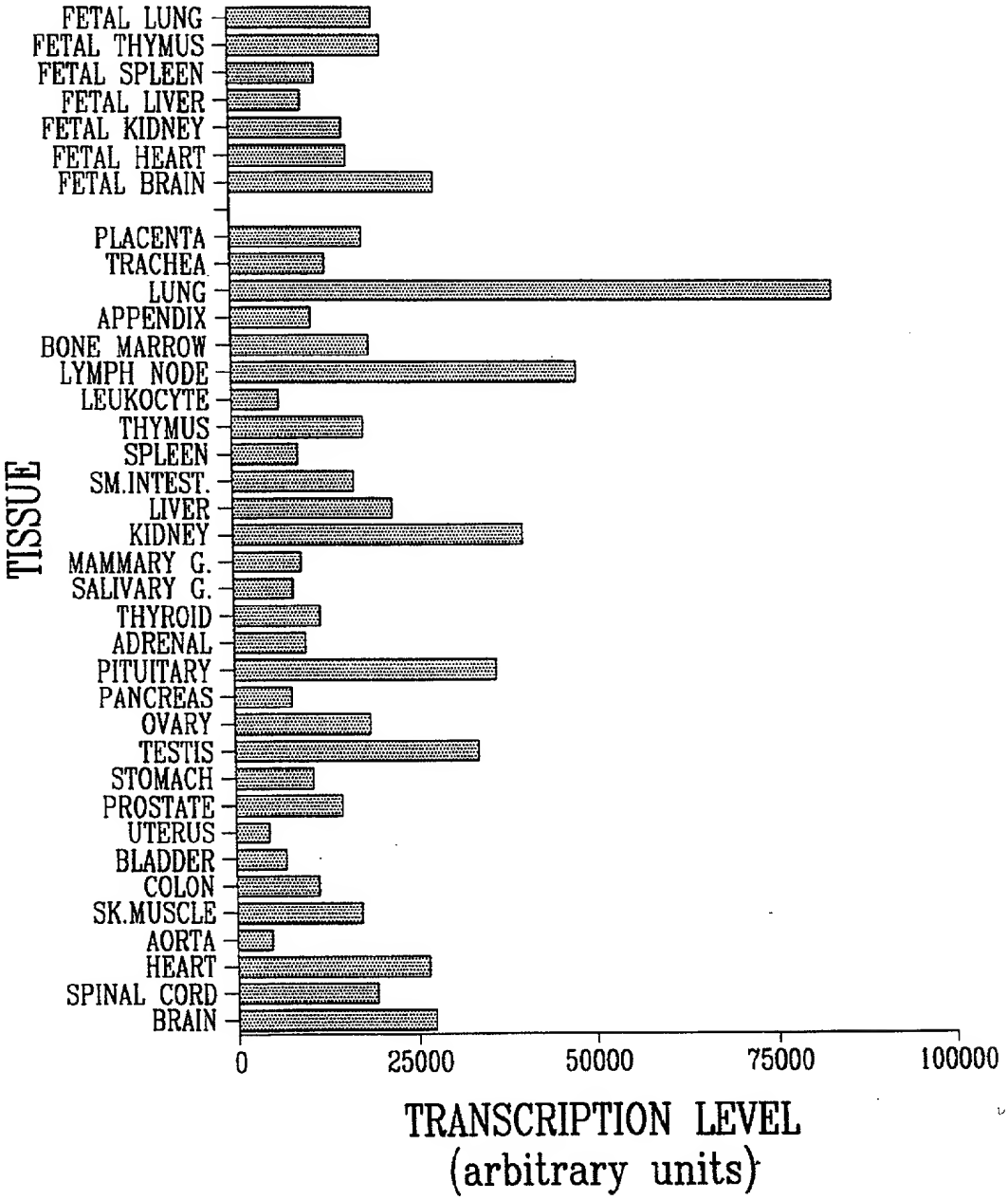
FETAL LUNG
FETAL THYMUS
FETAL SPLEEN
FETAL LIVER
FETAL KIDNEY
FETAL HEART
FETAL BRAIN
PLACENTA
TRACHEA
LUNG
APPENDIX
BONE MARROW
LYMPH NODE
LEUKOCYTE
THYMUS
SPLEEN
SM.INTEST.
LIVER
KIDNEY
MAMMARY G.
SALIVARY G.
THYROID
ADRENAL
PITUITARY
PANCREAS
OVARY
TESTIS
STOMACH
PROSTATE
UTERUS
BLADDER
COLON
SK.MUSCLE
AORTA
HEART
SPINAL CORD
BRAIN
TISSUE
0
25000
50000
75000
100000
TRANSCRIPTION LEVEL
(arbitrary units)

Fig. 6

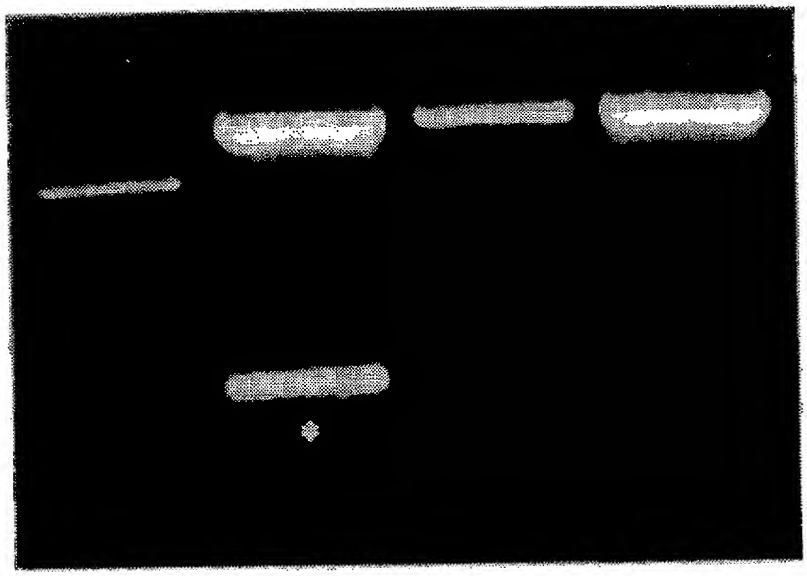

Fig. 7

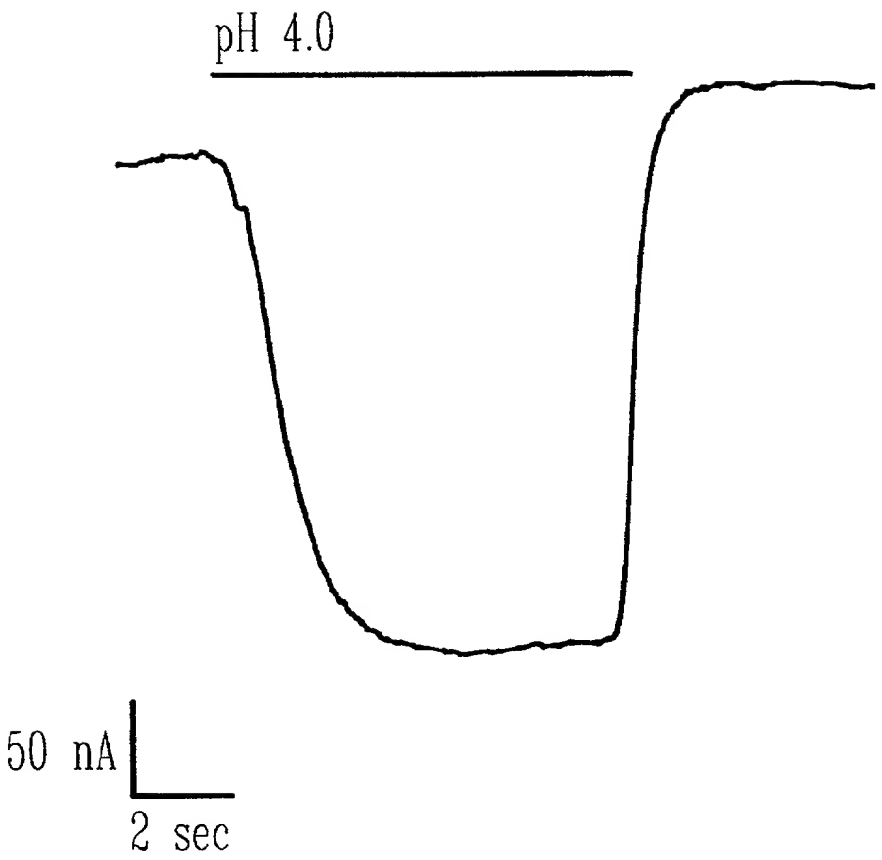

FIG. 8

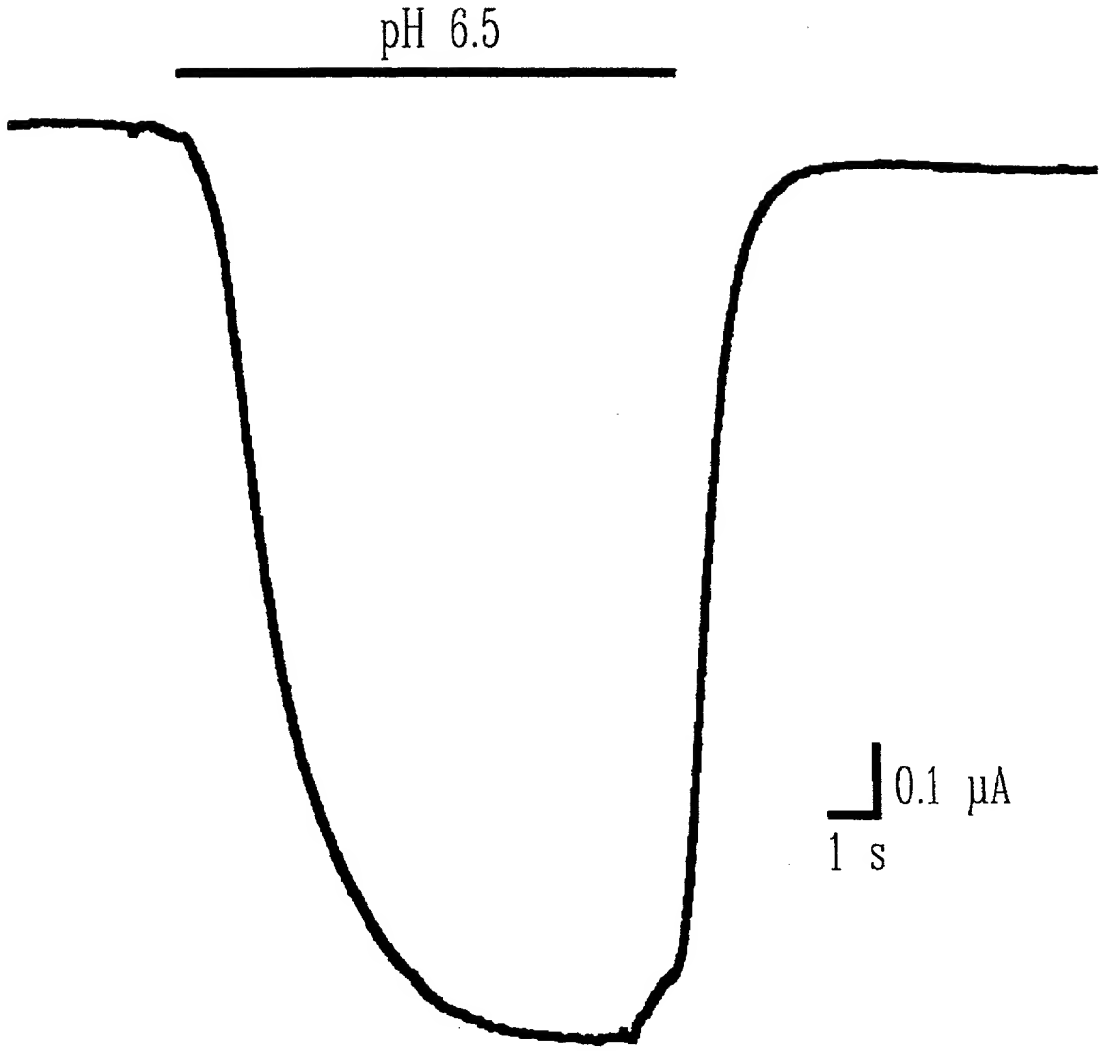

Fig. 9

Docket No.

Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

DNA ENCODING A HUMAN PROTON-GATED ION CHANNEL AND USES THEREOF

the specification of which

(check one)

☐ is attached hereto.

☒ was filed on **29 October 1998** as United States Application No. or PCT International Application Number **PCT/CA98/01016**

and was amended on **N/A**

(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)			Priority Not Claimed
2,219,713	**CA**	**29 October 1997**	☐
(Number)	(Country)	(Day/Month/Year Filed)	
			☐
(Number)	(Country)	(Day/Month/Year Filed)	
			☐
(Number)	(Country)	(Day/Month/Year Filed)	

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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